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Henstra, S.A.; Tuinhof, M.; Duurkens, R.H.T.; Robillard, G.T.

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# The *Bacillus stearothermophilus* Mannitol Regulator, MtlR, of the Phosphotransferase System

A DNA-BINDING PROTEIN, REGULATED BY HPr AND IICB<sup>mtl</sup>-DEPENDENT PHOSPHORYLATION\*

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Sytse A. Henstra, Menno Tuinhof, Ria H. Duurkens, and George T. Robillard‡

From the Departments of Biochemistry, the Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

D-Mannitol is taken up by *Bacillus stearothermophilus* and phosphorylated via a phosphoenolpyruvate-dependent phosphotransferase system (PTS). The genes involved in the mannitol uptake were recently cloned and sequenced. One of the genes codes for a putative transcriptional regulator, MtlR. The presence of a DNA binding helix-turn-helix motif and two antiterminator-like PTS regulation domains, suggest that MtlR is a DNA-binding protein, the activity of which can be regulated by phosphorylation by components of the PTS. To demonstrate DNA binding of MtlR to a region upstream of the mannitol promoter, by DNA footprinting, MtlR was overproduced and purified. EI, HPr, IIA<sup>mtl</sup>, and IICB<sup>mtl</sup> of *B. stearothermophilus* were purified and used to demonstrate that MtlR can be phosphorylated and regulated by HPr and IICB<sup>mtl</sup>, *in vitro*. Phosphorylation of MtlR by HPr increases the affinity of MtlR for its binding site, whereas phosphorylation by IICB<sup>mtl</sup> results in a reduction of this affinity. The differential effect of phosphorylation, by two different proteins, on the DNA binding properties of a bacterial transcriptional regulator has not, to our knowledge, been described before. Regulation of MtlR by two components of the PTS is an example of an elegant control system sensing both the presence of mannitol and the need to utilize this substrate.

Many bacteria transport D-mannitol and other carbohydrates via a phosphoenolpyruvate-dependent phosphotransferase system (PTS)<sup>1</sup> (1–3). Two general cytoplasmic proteins, EI and HPr, are responsible for the transfer of the phosphoryl group from PEP to different sugar-specific PTS proteins. Each sugar-specific system consists of three parts: IIA, IIB, and IIC. IIC is the transmembrane transporter, responsible for the transfer of the sugar across the cytoplasmic membrane. The transported sugar is phosphorylated by IIB while it is still

bound to IIC. IIA is responsible for the transfer of the phosphoryl group from HPr to IIB. Different fusions between IIA, IIB, and IIC occur naturally in the PTS. For example, in the mannitol uptake system of *Escherichia coli*, these proteins are covalently linked as one polypeptide chain, IICBA<sup>mtl</sup>, whereas in *B. stearothermophilus*, the same system consists of a soluble IIA<sup>mtl</sup> and a membrane-bound IICB<sup>mtl</sup>.

Recently, the entire mannitol operon of *Bacillus stearothermophilus* was cloned (4). Four open reading frames, *mtlA*, *mtlR*, *mtlF*, and *mtlD*, were identified within the operon, coding for the mannitol transporter (IICB<sup>mtl</sup>), a putative transcriptional regulator (MtlR), enzyme IIA<sup>mtl</sup>, and the mannitol-1-phosphate dehydrogenase (MPDH), respectively (Fig. 1). The mannitol transporter IICB<sup>mtl</sup> was expressed, and its involvement in the uptake of mannitol by *B. stearothermophilus* was confirmed (4). The sequence of the *mtlR* gene resembles that of transcriptional regulators, such as antiterminators and repressors, and is, therefore, expected to be involved in the regulation of the mannitol operon. In this paper, we report the functional expression and isolation of the *B. stearothermophilus* EI, HPr, IIA<sup>mtl</sup>, and MtlR and the analysis of the *mtlR* gene product, MtlR, that reveals its function as a transcriptional regulator, involved in the regulation of the mannitol operon.

## MATERIALS AND METHODS

Restriction enzymes, *Taq*-DNA polymerase, nucleotides, oligonucleotide-kinase, pyruvate-kinase, IPTG, 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside, and the first strand cDNA synthesis kit for reverse transcription-PCR (Avian Myeloblastosis Virus) were purchased from Boehringer Mannheim. [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol) and [<sup>14</sup>C]mannitol (50–62 mCi/mmol) were obtained from Amersham, Ni-NTA agarose from Qiagen, and PET15b from Novagen. RNase-free RQ1-DNase I was obtained from the Promega core footprinting system. Thrombin, with a specific activity of 8165 units/mg of protein, was from ICN Biomedicals Inc. Primers were synthesized by Eurosequence. PEP and yeast tRNA were purchased from Sigma. The vectors pSK<sup>+</sup>, pET15-B, and pGEX-2T were obtained from Stratagen, Novagen, and Amersham Pharmacia Biotech, respectively. All DNA manipulations and the expression of *B. stearothermophilus* IIA<sup>mtl</sup> were performed in *E. coli* JM101 (5). The MtlR gene was overexpressed in *E. coli* B121(DE3) (6). The *B. stearothermophilus* mannitol transporter, IICB<sup>mtl</sup>, was expressed in the mannitol deletion *E. coli* strain LGS322 (7), as described by Henstra *et al.* (4), and the *B. stearothermophilus* EI and HPr were expressed in *E. coli* ZSC112 (8).

**General Methods**—DNA was isolated from agarose gels using the Qiagen gel extraction kit. Protein concentrations were determined according to Bradford (9). General DNA manipulations were performed as described in Sambrook *et al.* (10). Sequence data base searches were performed using the program BLAST at the National Center for Biotechnology Information (11).

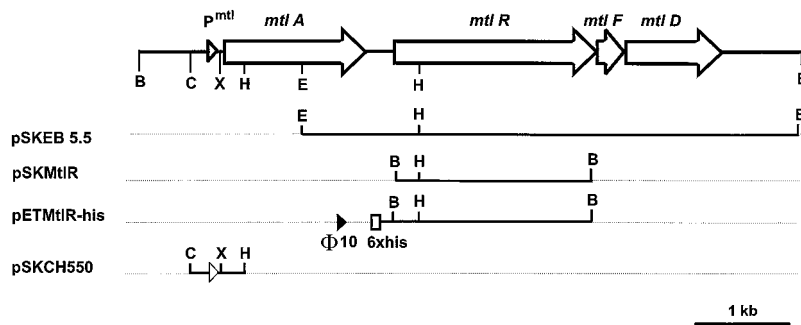
**Construction of pETMtlR-his**—Overexpression of the *mtlR* gene was established with the T7<sup>+</sup> expression vector pET15-b, which enables a thrombin cleavable histidine-tag to be fused to the N terminus of the protein. To enable the insertion of the *mtlR* gene into pET15-b, a *Bam*HI restriction site was created at the start and downstream of the MtlR gene, with PCR using two mutagenic primers (N terminus, 5'-

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‡ To whom correspondence should be addressed. Fax: 31-0-503634429; E-mail: G.T.Robillard@chem.rug.nl

<sup>1</sup> The abbreviations used are: PTS, phosphoenolpyruvate-dependent phosphotransferase system; PEP, phosphoenolpyruvate; EI, enzyme I of the PTS; mtl, mannitol; IICB<sup>mtl</sup>, mannitol permease; HPr, histidine phosphocarrier protein; IIA<sup>mtl</sup>, enzyme IIA of the mannitol PTS; MPDH, mannitol-1-phosphate dehydrogenase; IPTG, isopropyl- $\beta$ -D-thiogalactopyranoside; PCR, polymerase chain reaction; GST, glutathione S-transferase; HTH, helix-turn-helix; PRD, PTS regulation domain; CRE, catabolite response element; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol.

FIG. 1. Schematic overview of the mannitol operon and subclones used to create the expression vector pET-MtlR-his and the subclones involved in the DNA footprint, promoter activity, and primer extension experiments. The genes of the mannitol operon, the mannitol and T7 virus  $\Phi$ 10 promoter, and the His tag are indicated by open arrows, open and filled triangles, and open box, respectively. Restriction enzymes *Bam*HI, *Cla*I, *Eco*RI, *Hind*III, *Sal*I, and *Xba*I are indicated by B, C, E, H, S, and X, respectively.



GGG GCA ATG GAT CCA TCT GCA CGC-3'; C terminus, 5'-AGG AGT GAA TGA CAT GTG GAT CCC AAT TT-3'). The PCR was performed in a total volume of 100  $\mu$ l with 10 mM Tris-HCl, pH 8.0, 5 mM MgCl<sub>2</sub>, 100 mM NaCl, 1 mM  $\beta$ -mercaptoethanol, 1 mM dNTPs, 1  $\mu$ g of each primer, and 2.5 units of Pwo-DNA polymerase. 100–500 pg of pSKEB5.5 was added as template DNA. This plasmid contains a 5.5-kilobase pair DNA fragment of the mannitol operon, including the entire *mtlR* gene (4). After 25 cycles comprising 1 min of denaturation at 94 °C, 1 min of primer annealing at 30 °C, and 2 min of extension at 72 °C, the PCR product was separated by agarose gel electrophoresis. Fragments of 2116 bp were isolated from the gel, cut with *Bam*HI, and ligated into the *Bam*HI site of pSK<sup>+</sup> to create pSKMtlR (Fig. 1). Positive clones were identified by blue/white screening on IPTG, 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside, ampicillin plates. The sequence of the insert of one of the positive clones was checked by sequencing. The *MtlR* gene in pSKMtlR was ligated into the *Bam*HI site of pET15b after digestion with *Bam*HI creating the expression vector pETMtlR-his.

**Expression and Isolation of MtlR-his**—A preculture of BL21-DE3 with pETMtlR-his was grown overnight at 30 °C and was diluted 100-fold in 5-liter flasks with 0.5 liters of LB medium (10 g of trypton, 5 g of yeast extract, and 10 g of NaCl per liter). The cultures were grown at 30 °C with vigorous shaking at 300 rpm. At  $A_{660} = 0.6$ , the culture was induced with 0.8 mM IPTG and grown for 90 min, after which the cells were collected by centrifugation (3000  $\times g$  for 10 min at 4 °C). The pellet was washed in 0.5 liter of 25 mM Tris, pH 8.0, and 0.2 M KCl and resuspended in 20 ml of 25 mM Tris, pH 8.0, 1 M NaCl, 3 mM  $\beta$ -mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 1 mM MgCl<sub>2</sub>, and 0.2 mg/ml DNase and RNase per g of cells. The cells were ruptured with a French press at 10,000 psi, and 2 mM EDTA was added. Whole cells, cell debris, and precipitate were removed by centrifugation at 3000  $\times g$  for 10 min; membranes were removed by centrifugation at 200,000  $\times g$  for 30 min. 1 ml of Ni-NTA resin per g of cells and 2 mM phenylmethylsulfonyl fluoride were added to the supernatant, and the mixture was incubated with continuous agitation for 30 min at 4 °C. The Ni-NTA resin was collected in a column and subsequently washed with 10 column volumes of wash buffer (25 mM Tris-HCl, pH 8, 1 M NaCl, 3 mM  $\beta$ -mercaptoethanol, and 40 mM imidazole). The protein was eluted by resuspension of the resin in 5–10 ml of wash buffer containing 200 mM imidazole. After removal of the Ni-NTA resin by filtration, 15 mM EDTA was added, and the purified protein was dialyzed twice against 40 volumes of 25 mM Tris-HCl, pH 8, 0.2 M KCl, and 3 mM  $\beta$ -mercaptoethanol.

**Expression and Purification of EI and HPr of *B. stearotheophilus***—EI and HPr of *B. stearotheophilus* were expressed from the plasmid pLOI1801 in the EI, HPr deletion mutant *E. coli* ZSC-112 as described by Lai and Ingram (12). A culture of ZSC-112 containing pLOI1801 was grown overnight at 37 °C, after which the cells were collected by centrifugation. The pellet was washed in 10 mM Tris, pH 7.5, and 1 mM DTT and resuspended in 10 mM Tris, pH 7.5, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 mM MgCl<sub>2</sub>, and 0.2 mg/ml DNase and RNase. The cells were ruptured with a French press at 10,000 psi, and 2.5 mM EDTA was added. Whole cells and precipitates were removed by centrifugation at 20,000  $\times g$ . The supernatant was dialyzed against 10 mM Tris-HCl, pH 7.5, and 2 mM DTT for 16 h and applied to a 40-ml Q-Sepharose column. The column was washed with 2 column volumes of Bis-Tris, pH 7.0, 1 mM DTT, and the proteins were eluted with a 400-ml linear gradient from 1 to 600 mM NaCl in buffer. HPr eluted in the wash step and was purified to homogeneity by gel filtration on a G75 column. The EI eluted at 350 mM NaCl and was directly used in the further experiments, although some impurities were still present.

**Expression and Purification of IIA<sup>mtl</sup> of *B. stearotheophilus***—IIA<sup>mtl</sup> was expressed as a fusion product with glutathione S-transferase

(GST) using the plasmid pGEX-2T. To enable the insertion of the *mtlF* gene into pET15-b, a *Bgl*II and an *Eco*RI restriction site were created, by PCR, at the start and downstream of the *mtlF* gene, respectively. The PCR was performed as described for the construction of pETMtlR-his using the same template but with two other mutagenic primers (N terminus, 5'-GTG AAT GAC AGA TCT ATG CCA ATT-3'; C terminus, 5'-GGA ATG AAT TCC TAA ACA TGC-3'). PCR fragments of 478 bp were isolated from the gel and ligated into the *Eco*RV site of pSK<sup>+</sup> to create pSKMtlF. The sequence of the insert of one of the positive clones was checked by sequencing. The *mtlF* gene in pSKMtlF was ligated into the *Bam*HI and *Eco*RI sites of pGEX-2T after digestion with *Bgl*II and *Eco*RI creating the expression vector pGEXIIA.

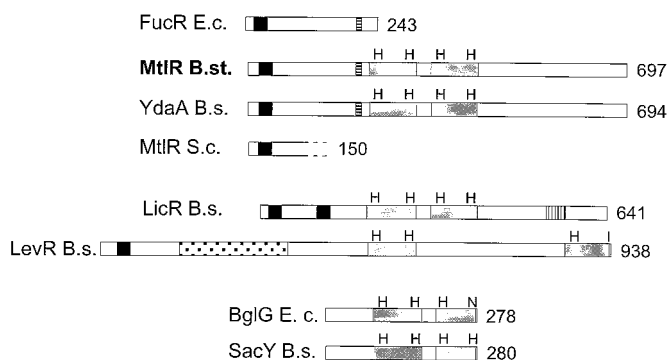
For the expression of the GST-IIA<sup>mtl</sup> fusion product, *E. coli* Jm101 with pGEXIIA was grown to  $A_{660} 0.85$ , induced with 0.5 mM IPTG, and grown overnight. 4 g of cells were collected by centrifugation at 6000  $\times g$ , washed in 50 mM NaPi, pH 7.5, resuspended in 20 ml of 50 mM NaPi, pH 7.5, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 mM MgCl<sub>2</sub>, and 0.2 mg/ml DNase and RNase. After disruption with a French press, whole cells and precipitates were removed by centrifugation at 20,000  $\times g$  followed by the addition of 2.5 mM EDTA, 150 mM NaCl, and 2.4 g (dry weight) of glutathione agarose equilibrated in 50 mM Tris, pH 7.5, and 150 mM NaCl. The mixture was incubated with continuous agitation for 1 h at room temperature and collected in a column. Unbound protein was removed by washing with 10 column volumes of equilibration buffer. The column material was resuspended in 50 ml of equilibration buffer containing 0.3 mM CaCl<sub>2</sub> and 150 mg of thrombin and incubated overnight at room temperature. Soluble IIA<sup>mtl</sup>, cleaved from the bound GST, was collected by filtration. Thrombin, also present in the filtrate, was precipitated by incubating the sample for 30 min at 60 °C. Pure IIA<sup>mtl</sup> was then obtained by centrifugation.

**Phosphorylation of MtlR**—[<sup>32</sup>P]PEP was synthesized, following the method of Roossien *et al.* (13). *B. stearotheophilus* IICB<sup>mtl</sup>-containing membrane vesicles and purified IICB<sup>mtl</sup> were created as described by Henstra *et al.* (4). MtlR, enzymes, and vesicles were diluted in phosphorylation buffer (25 mM Tris-HCl, pH 7.5, 0.5 mM MgCl<sub>2</sub>, 1 mM NaF, and 5 mM DTT). The reaction was started by adding [<sup>32</sup>P]PEP and stopped, after incubation at 30 °C, with 1/2 volume denaturation buffer. Following separation of the proteins on a 15% SDS-polyacrylamide gel by electrophoresis, phosphorylation of proteins was visualized on a Molecular Dynamics PhosphorImager 425. The autoradiogram was analyzed using the Image Quant program.

**DNA Footprint**—A single-end <sup>32</sup>P-labeled DNA probe of the mannitol promoter region was synthesized in a PCR in which one of the primers was labeled. 28.5 pmol of the forward primer sah1 (5'-GGC AGG TGA ATT GTT AAA G-3') priming at position -127 to -109 was labeled with 100  $\mu$ Ci of [<sup>32</sup>P]ATP (3000 Ci/mmol) by T4-polynucleotide kinase as described by Boehringer Mannheim. The labeled primer was purified by chloroform/phenol and chloroform extractions followed by an ethanol precipitation. 19 pmol of the labeled forward primer was built into a 473-bp probe by PCR in a mixture containing 10 mM Tris, 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 200  $\mu$ M dNTPs, 2.5 units of *Taq* DNA polymerase, 57 pmol of universal reverse primer (5'-ACAGGAAACAGCTATGACC-3') and 1 ng of template DNA. The pSK-derived subclone pSKCH550, containing the area of the mannitol promoter from *Cla*I (position -354) to *Hind*III (position +212), was used as template DNA. After 30 cycles of 1 min of denaturation at 94 °C, 1 min of annealing at 55 °C, and 1 min of elongation at 72 °C, the 473-bp PCR product was separated by electrophoresis on a 0.8% agarose gel and isolated from the gel with a gel extraction kit from Qiex.

The binding of MtlR to the target DNA was achieved by mixing the sample with 25  $\mu$ l of binding buffer (20 mM Tris-HCl, 80 mM KCl, 25% glycerol, 8 mM MgCl<sub>2</sub>, and 2 mM DTT, pH 8.0) and triple distilled water to a final volume of 46  $\mu$ l. Each reaction was started by adding 4  $\mu$ l of





**FIG. 2. Schematic presentation of the topology of the sequence of several transcriptional regulators similar to MtlR.** Shown are the fucose repressor of *E. coli* FucR (P11554); the putative mannitol regulators MtlR (U18943) of *B. stearotheophilus*, YdaA (P96574) of *B. subtilis*, and the N terminus of the MtlR of *S. carnosus* (P28008); the transcriptional regulators LicR (previously called CelR) (P46321) and LevR (P23914) of *B. subtilis*; and the antiterminators BglG (P11989) and SacY (P15401) of *E. coli* and *B. subtilis*, respectively (All accession numbers are from Swiss. Prot. except Vidgy3 which can be obtained at GenBank). The distinct domains found in MtlR with similarities to the other listed proteins are displayed as a black box for the DNA binding HTH motif, a horizontally striped box for the putative inducer binding sites, and two gray boxes representing the PTS regulation domains PRD-I and PRD-II. The  $\sigma$ -factor binding domain, found only in LevR, is indicated by the dotted box, and a IIA<sup>mtl</sup>-like domain, found only in LicR, is indicated by the vertically striped box. The positions of the conserved histidines, probably involved in phosphorylation, are indicated by H. Deviations of one of the conserved histidine in LevR and BglG to an isoleucine and an aspartate are indicated by I and N, respectively.

20–40 kcpm DNA probe (~50 fmol of DNA) to the mixture, followed by an incubation of 15 min at 30 °C. The DNA was digested by adding 50  $\mu$ l of 10 mM Tris, pH 8.0, 10 mM MgCl<sub>2</sub>, 5 mM CaCl<sub>2</sub>, 0.03 units/ $\mu$ l of RQ1-DNaseI and incubated for 2 min at room temperature. The digestion was stopped with 90  $\mu$ l of 0.6 M NaAc, 30 mM EDTA, 0.5% SDS, and 30  $\mu$ g/ $\mu$ l yeast tRNA. The digest was then purified by a chloroform phenol extraction and precipitated with 2.5 volumes of EtOH at –20 °C. Samples were separated on a 5% sequencing gel. A reference sequence ladder was generated by PCR as described by Krishnan *et al.* (14) with the same labeled primer and template used for the synthesis of the probe.

**Primer Extension**—Samples of total RNA were isolated from *E. coli* or *B. stearotheophilus* grown on minimal medium supplemented with 0.5% glucose, mannitol, or mannitol plus glucose. Exponentially growing cells were harvested at an A<sub>660</sub> of 0.5 and RNA was extracted as described by Ausubel *et al.* (15). For the primer extension reaction, 10 pmol of primer sah2 (3'-GACCGAAAGCACATTCTAAGTCGCAAAAC-5'), at position +106 to +82, was labeled with 30  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP (3000 Ci/mmol) by T4-polynucleotide kinase as described by Boehringer Mannheim. Each reaction was performed with 10  $\mu$ g of total RNA and 500 kcpm of primer, according to the manual of the Boehringer Mannheim first strand cDNA synthesis kit for reverse transcription-PCR. The samples were analyzed on a sequencing gel together with a sequencing reaction performed with the same labeled primer.

**Phosphoenolpyruvate-dependent Mannitol Phosphorylation Assay**—PEP-dependent mannitol phosphorylation activity was measured as described by Robillard and Blaauw (16). The assays were performed in a 100- $\mu$ l assay mixture containing 25 mM Tris, pH 7.5, 5 mM MgCl<sub>2</sub>, 0.25% decyl-polyethylene glycol, 5 mM DTT, 5 mM PEP, 33  $\mu$ M [<sup>14</sup>C]mannitol and 0.04 mg/ml EI, 18  $\mu$ M HPr and 24  $\mu$ M IIA<sup>mtl</sup>, all from *B. stearotheophilus*.

**Mannitol-1-phosphate Dehydrogenase Assay**—MPDH activity was measured as described by Novotny *et al.* (17). The sample was incubated 3 min in 70 mM Tris-HCl (pH 9) and 1 mM NAD<sup>+</sup> at 20 °C. Conversion of NAD<sup>+</sup> to NADH was followed for 7 min at 340 nm, after starting the reaction with 4 mM mannitol 1-phosphate.

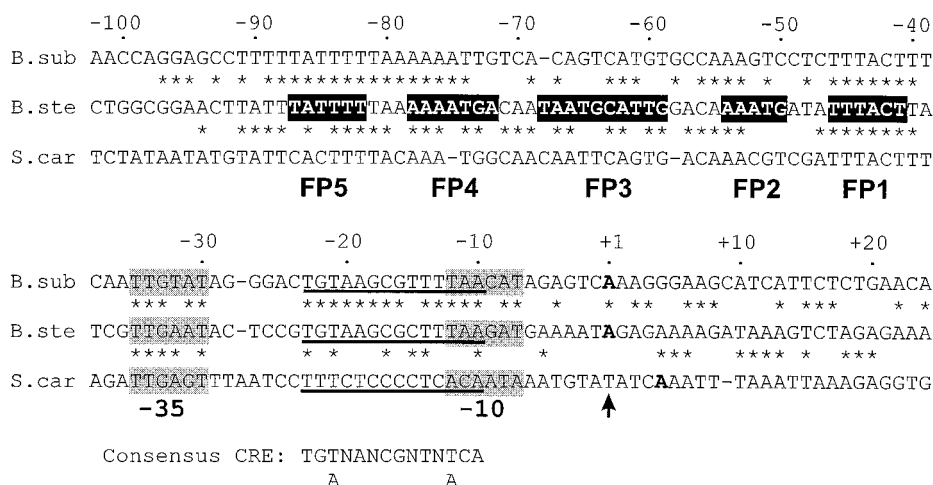
## RESULTS

**Sequence of the mtlR Gene**—The mtlR gene was cloned as part of the mannitol operon and sequenced as described by Henstra *et al.* (4). The gene of 2091 bp codes for a protein of 697 amino acids with a calculated mass of 79 kDa. In the protein

sequence, conserved areas are found that show resemblance to domains of two different types of transcriptional regulators (Fig. 2). First, a helix-turn-helix motif (Fig. 2, black box) was identified at the N terminus using the method described by Dodd and Egan (18), which shows similarity with the HTH motifs of members of the DeoR family of transcriptional regulators, such as the fucose (19), glucitol (20), and deoxyribonucleotide (21) repressor of *E. coli* and the lactose regulator of *Lactococcus lactis* (22) and of *Streptococcus mutans* (23). Sequence similarity to the rest of these regulators is low (10–18% overall identity), with the exception of a region that, in case of the DeoR and the LacR regulator, is involved in binding the inducer. These inducer binding sites are the targets of phosphorylated substrates, such as galactose 6-phosphate in the case of LacR of *L. lactis* (24). Second, the central part of the protein contains sequences similar to the C terminus of antiterminators of the BglG family, such as BglG (25) of *E. coli* and SacT (26), SacY (27), GlcT (28), and LicT (29) of *Bacillus subtilis*. Two homologous PTS regulation domains, PRD-I and PRD-II (Fig. 2, gray boxes), are located in this part of the protein. They appear to be involved in the regulation of the activity of the antiterminator via phosphorylation by PTS components (30–34). All four putative phosphorylation sites, histidines 235 and 294 in PRD-I and histidines 348 and 405 in PRD-II, and their surroundings are conserved in most of the antiterminators and MtlR, suggesting that MtlR could also be controlled by phosphorylation by EI and HPr and the mannitol-specific components IIA<sup>mtl</sup> and IICB<sup>mtl</sup>. Combinations of a DNA binding motif with an antiterminator-like motif have been found before for the levansase regulator LevR (35) and the lichean regulator LicR (36) of *B. subtilis*. The homology between MtlR and these proteins is limited to the regions involved in DNA binding and in phosphorylation by the PTS. Recently, the gene ydaA, discovered by the *B. subtilis* genome sequence project, was submitted to GenBank™ (accession number AB001488). The product of this gene has the same topology as the *B. stearotheophilus* MtlR and a 39% overall sequence identity; it is the protein most similar to MtlR found in the sequence data bases. The gene is not located near the mannitol operon of *B. subtilis*, but it could be involved in the regulation of this operon. Two other Gram-positive mannitol operons possess parts of genes that show similarity to MtlR. First, an open reading frame is found within the *S. mutans* mannitol operon that codes for a sequence similar to the C terminus of MtlR (37). Second, *Staphylococcus carnosus* possesses an open reading frame of 150 amino acids downstream of mtlA that contains a HTH motif with 52% identity compared with the HTH in MtlR.

**The Mannitol Promoter of *B. stearotheophilus***—The high homology between the above-mentioned proteins and the *B. stearotheophilus* MtlR suggest that the mannitol operons of these microorganisms can be regulated in a comparable way. A conserved sequence of 85 bases was revealed in the putative promoter regions upstream of the mtlA genes of *B. stearotheophilus*, *B. subtilis*, and *S. carnosus* (Fig. 3). The identity between these regions in *B. stearotheophilus* and *B. subtilis* or *B. stearotheophilus* and *S. carnosus* was 67 and 47%, respectively. A putative CRE sequence (38), involved in catabolite repression, is found in all three promoter regions. Deviations from the consensus CRE sequence (39) are found at one position for the *B. stearotheophilus* and *B. subtilis* and at three positions for the *S. carnosus* CRE box. A conserved putative  $\sigma^A$ -dependent –35 sequence was found 6–7 base pairs upstream of the CRE box for all three organisms (40). The accompanying –10 region was located 17 bp downstream of the –35 sequence, overlapping the CRE box in *B. stearotheophi-*

FIG. 3. Sequence similarity between the mannitol promoter region of *B. subtilis*, *B. stearotheophilus*, and *S. carnosus* and the location of the footprints shown in Fig. 8. Identical bases in the *B. subtilis* or *S. carnosus* and *B. stearotheophilus* promoter region are indicated by asterisks. The predicted  $-10$  and  $-35$  regions are indicated by gray boxes, and the CRE boxes matching the CRE consensus sequence are indicated by lines. The predicted transcriptional start sites are shown in boldface. The position of the experimentally determined transcriptional start of the *B. stearotheophilus* mannitol promoter is indicated by the arrow. The regions protected by MtlR against DNase I digestion are shown as black boxes and marked with FP1–FP5.



*lus* and *B. subtilis*. In *S. carnosus*, no clear  $-10$  sequence could be found at this position. Prediction of the transcription start point, by the method of Reese *et al.* (41), confirmed the *B. stearotheophilus* and *B. subtilis* promoter but suggested a 4-bp shift upstream for the *S. carnosus* promoter. The *B. stearotheophilus* transcription start was confirmed by primer extension using RNA isolated from *B. stearotheophilus* grown on mannitol, mannitol plus glucose, and glucose. The result is presented in Fig. 4 and shows that transcription is started at an adenine 7 bp downstream from the  $-10$  sequence.

**Expression and Purification of MtlR**—The MtlR gene was cloned in the T7 expression plasmid pET-15b (see under “Materials and Methods”), creating a fusion of a His tag and a thrombin cleavage site at the N terminus of MtlR. The protein was successfully expressed from the plasmid pETMtlR-his in *E. coli* BL21-DE3 (Fig. 5, lane 2). After disruption of the cells, part of the protein was found in an insoluble form and was removed by centrifugation (lane 3). The ratio of soluble to insoluble protein could be improved by lowering the temperature and increasing agitation during growth. Also, the addition of 1 M NaCl to the disruption buffer decreased the amount of precipitated protein in the crude extract. Most of the soluble His-tagged MtlR (Fig. 5, lane 4) was bound to the NTA Ni-agarose. After the column material was washed, the protein was eluted with a purity greater than 95% (Fig. 5, lane 5). The mass was determined to be 81,590 Da by matrix-assisted laser desorption ionization-time of flight mass spectroscopy. The calculated mass of the fusion product, 81,545 Da, was within the experimental error of the determined mass (data not shown). Analysis of the purified protein by Western blotting using His tag-directed antibodies (data not shown) revealed that the remaining impurities visible on Coomassie-stained SDS-PAGE are N-terminal degradation products of the MtlR protein. Serious protein degradation was observed during the early purifications. This problem was solved by harvesting cells before they enter the stationary growth phase.

**Purification of EI, HPr, and IIA<sup>mtl</sup> of *B. stearotheophilus***—*B. stearotheophilus* EI and HPr, expressed simultaneously in the EI and HPr deficient *E. coli* ZSC112, were separated from each other and from other proteins on a Q-Sepharose ion exchange column. The partly purified EI fraction (Fig. 5, lane 6) was not purified to homogeneity but used directly in subsequent experiments. HPr was purified to homogeneity by gel filtration (Fig. 5, lane 7).

The GST-IIA<sup>mtl</sup> fusion product could be easily separated from the other *E. coli* proteins by binding to glutathione agarose. IIA<sup>mtl</sup> was cleaved from the column bound GST by digestion with thrombin. The removal of thrombin by precipitation

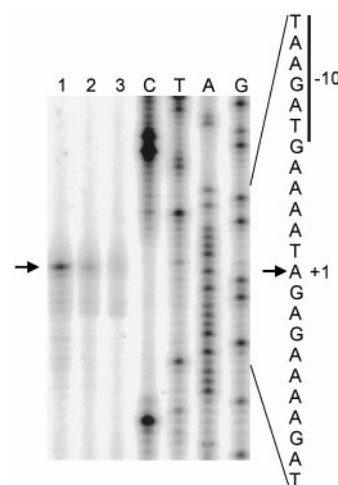


FIG. 4. Determination of the transcriptional start of the mannitol operon. Primer extensions products were obtained from RNA isolated from *B. stearotheophilus* grown on mannitol (lane 1), mannitol plus glucose (lane 2), and glucose (lane 3) as described under “Materials and Methods.” The sequence ladder was obtained with the same primer used for the primer extension reaction. On the right the position of the  $-10$  sequence, and the start site,  $+1$ , is indicated by an arrow.

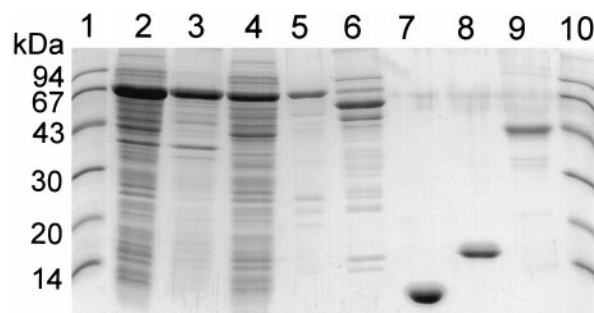


FIG. 5. Overview of the expression and isolation of *B. stearotheophilus* MtlR and the purified *B. stearotheophilus* PTS proteins EI, HPr, IIA<sup>mtl</sup>, and IICB<sup>mtl</sup> visualized by SDS-PAGE. Lanes 1 and 10, protein standards; lane 2, crude cell extract of BL21-DE3 with pETMtlR-his; lane 3, insoluble MtlR and protein removed by centrifugation; lane 4, the soluble fraction; lanes 5–9, the purified MtlR, EI, HPr, IIA<sup>mtl</sup>, and IICB<sup>mtl</sup>, respectively. Samples were analyzed on a 15% SDS-polyacrylamide gel, and the gel was stained with Coomassie Blue.

at 60 °C did not affect the activity of the purified IIA<sup>mtl</sup>. This procedure yielded a homogenous IIA<sup>mtl</sup> preparation (Fig. 5, lane 8). The N terminus of IIA<sup>mtl</sup> was sequenced, which con-

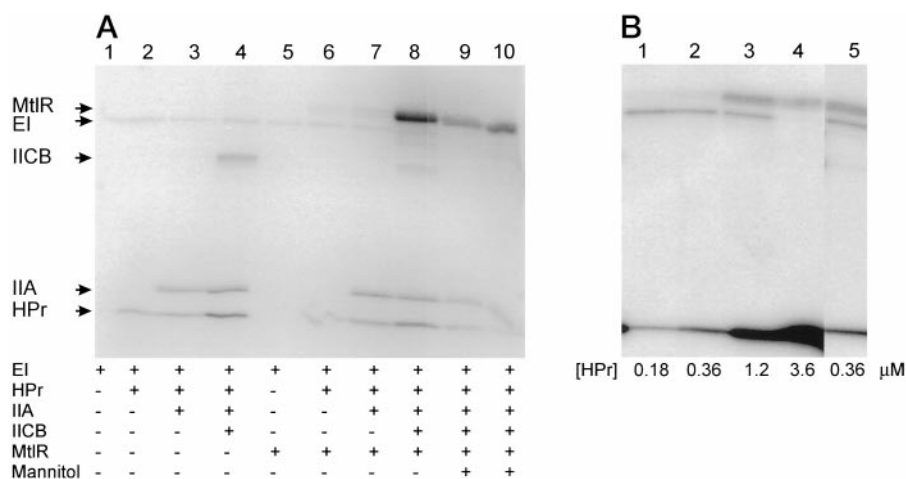


FIG. 6. *In vitro* phosphorylation of MtlR by proteins of the PTS. A, different combinations of components of the *B. stearotherophilus* PTS; EI, HPr, IIA<sup>mtl</sup>, and IICB<sup>mtl</sup> and MtlR were added as indicated at the bottom. Mannitol was added during the full experiment (lane 9) or in the last 1 min (lane 10). B, phosphorylation of MtlR by different amounts HPr (lanes 1–4) or a 6.7-fold increased in incubation time (lane 5). The concentrations MtlR, IIA<sup>mtl</sup>, and mannitol, when added, were 0.8 μM, 0.08 μM, and 10 mM, respectively. IICB<sup>mtl</sup> was added as LGS322 vesicles to a total protein concentration of 0.12 mg/ml. In A, the EI and HPr concentration were 1.5 μg/ml and 0.36 μM, respectively. In B, the EI concentration was 4 μg/ml, and the HPr concentration was as indicated under each lane. All reactions were started with 4.8 μM [<sup>32</sup>P]PEP. The mixtures were incubated at 30 °C for 15 min, with exception of lane 5 in panel B, in which the sample was incubated for 100 min. The reactions were stopped with denaturation buffer and loaded on a 15% SDS-PAGE gel. The difference in intensity between A and B is due to the fact that these data are taken from different experiments employing different exposure times.

firmed the sequence GSMPIKKE, which was expected after digestion of the fusion product by thrombin. The masses of HPr and IIA<sup>mtl</sup> were determined by matrix-assisted laser desorption ionization-time of flight mass spectroscopy to be 9032.5 and 15,958 Da, respectively. They correspond well to the theoretical masses of HPr without the first methionine (9035 Da) and IIA<sup>mtl</sup> with the expected replacement of the first methionine by a glycine (15,950 Da).

**Phosphorylation of MtlR by HPr and IICB<sup>mtl</sup>**—Purified MtlR was phosphorylated in the presence of partially purified components of the mannitol PTS, with exception of IICB<sup>mtl</sup>, which was added as inside-out *E. coli* LGS322 vesicles expressing *B. stearotherophilus* IICB<sup>mtl</sup>. PEP-dependent protein phosphorylation was performed with [<sup>32</sup>P]PEP and was visualized with a Phosphorimager after separation by SDS-PAGE (Fig. 6). The phosphorylation characteristics of MtlR were investigated by adding, one by one, the successive components of the PTS in the presence (Fig. 6A, lanes 5–8) and absence (lanes 1–4) of MtlR. Phosphorylation of the regulator was most obvious if all of the components of the *B. stearotherophilus* mannitol PTS, EI, HPr, IIA<sup>mtl</sup>, and IICB<sup>mtl</sup> were added (lane 8). Phosphorylation of MtlR by EI and HPr alone was also observed, although the amount phosphorylated MtlR was much lower than was found by phosphorylation via IICB. Phosphorylation by HPr became more evident when the HPr concentration was raised from 0.18 to 3.6 μM (Fig. 6B, lanes 1–4) or when the reaction time was increased from 15 to 100 min (Fig. 6B, lane 5). Addition of EI alone or IIA<sup>mtl</sup> to the EI/HPr mixture did not affect the phosphorylation level of MtlR. We therefore concluded that MtlR can be phosphorylated by both HPr and IICB<sup>mtl</sup>. The presence of the substrate mannitol in the reaction mixture during the entire incubation period reduced the amount of phosphorylated MtlR (Fig. 6A, lane 9). Addition of mannitol in only the last 1 min of the incubation period also resulted in a reduced level of phosphorylated MtlR (Fig. 6A, lane 10). This suggests that the phosphorylation of MtlR by IICB<sup>mtl</sup> is reversible.

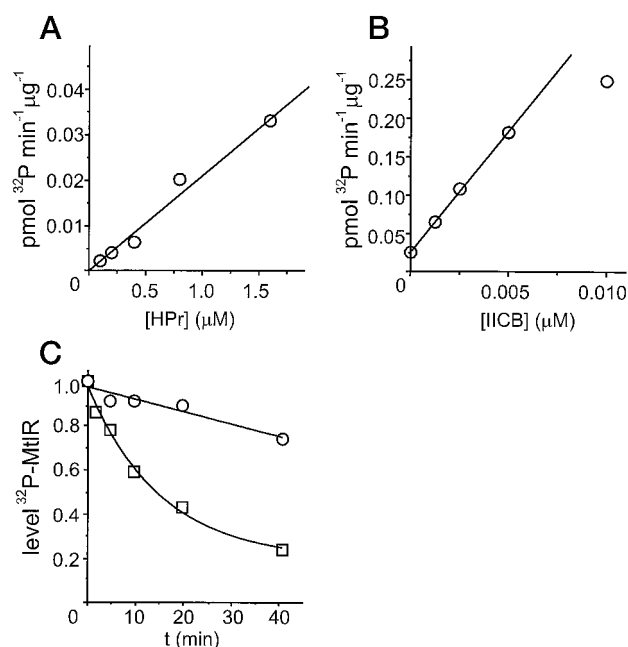
It is obvious from a comparison of Fig. 6A and Fig. 6B that a much higher concentration of HPr *versus* IICB<sup>mtl</sup> is necessary to get comparable levels of MtlR phosphorylation. In order to quantify this difference, the rate of MtlR phosphorylation was determined by following the time dependence of the phospho-

rylation levels of the MtlR band (see Fig. 7, A and B). The HPr and IICB<sup>mtl</sup> dependence of the MtlR phosphorylation rate was calculated from the linear portion of the plot to be 0.021 and 31 pmol min<sup>-1</sup> μg<sup>-1</sup> μM<sup>-1</sup>, respectively. The difference of a factor of 1470 between these dependences indicates that MtlR is much more rapidly phosphorylated by IICB<sup>mtl</sup> than by the same concentration of HPr.

To prove reversible phosphorylation of MtlR by IICB<sup>mtl</sup>, MtlR was phosphorylated under conditions where the protein is mainly phosphorylated by IICB<sup>mtl</sup> and purified on Ni-NTA agarose. <sup>32</sup>P-MtlR was mixed IICB<sup>mtl</sup> and incubated in the presence and absence of mannitol. The decrease in the amount of phosphorylated MtlR was followed against time with SDS-PAGE, quantified using the phosphorimager, and plotted Fig. 7C. The half-time of the phosphorylated MtlR is 15 and 119 min in the presence and absence of mannitol, respectively. This demonstrates that MtlR can be dephosphorylated by IICB<sup>mtl</sup> and mannitol.

**Binding MtlR to the Mannitol Promoter**—Footprint experiments were performed to determine whether MtlR is a DNA-binding protein and to locate the possible binding site. Based upon the homology between the promoters of *B. subtilis*, *S. carnosus*, and *B. stearotherophilus*, we expected that the region between -90 and -35 would contain the MtlR binding site. A DNA fragment, containing the sequence from position -127 to +212, was labeled at the -127 end as described under "Materials and Methods." This probe was incubated with different amounts of the purified MtlR and exposed to DNase I digestion. Five protected areas were observed, extending from position -41 to -86, in the presence of MtlR (Fig. 8). These areas are indeed located in the conserved regions in *B. subtilis*, *S. carnosus*, and *B. stearotherophilus*, just upstream of the mannitol promoter (Fig. 3). Hypersensitivity to DNase I digestion, induced by the presence of MtlR, was found at positions -70 and -48. The most obvious hypersensitivity was found at position -70 in the presence of 0.74 μM MtlR. The decrease of intensity of this hypersensitive band at the highest MtlR concentrations could be due to the tendency of the protein to aggregate, which might result in covering even the hypersensitive regions. The binding equilibria were calculated for each of the five protected regions (data not shown). The binding

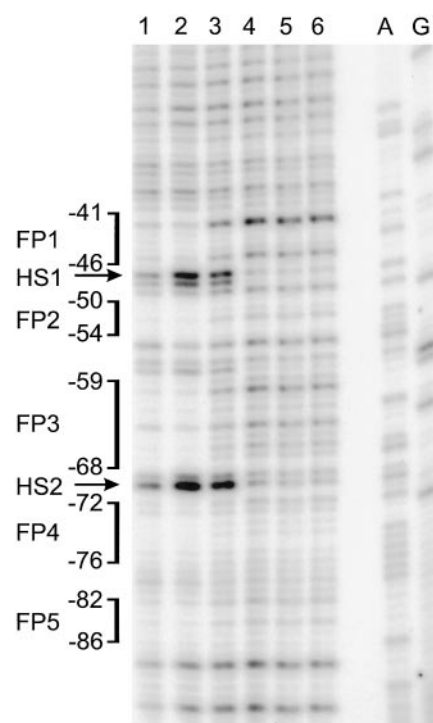




**FIG. 7. Phosphorylation and dephosphorylation of *B. stearotherophilus* MtlR.** The HPr-dependent (A) and IICB<sup>mtl</sup>-dependent (B) phosphorylation rates of MtlR were obtained from the linear relation between the incubation time and the intensity of the  $^{32}\text{P}$ -MtlR band on SDS-PAGE and were expressed as the amount  $^{32}\text{P}$ -MtlR formed per min per  $\mu\text{g}$  added MtlR. C, time-dependent dephosphorylation of  $^{32}\text{P}$ -MtlR by IICB<sup>mtl</sup> with (squares) and without (circles) mannitol. Phosphorylation of MtlR by various amounts of HPr was performed in 25 mM Tris-HCl, pH 7.5, 0.5 mM MgCl<sub>2</sub>, 5 mM DTT, 1 mM NaF, 4  $\mu\text{g}/\text{ml}$  EI, and 6.3  $\mu\text{M}$  [ $^{32}\text{P}$ ]PEP at 30 °C. Reactions were started by the addition of 0.8  $\mu\text{M}$  MtlR. Samples were taken at various times and analyzed by SDS-PAGE. Phosphorylation by IICB<sup>mtl</sup> was performed under conditions similar to those used in the phosphorylation by HPr except that IICB<sup>mtl</sup>, 0.4  $\mu\text{M}$  HPr, 0.4  $\mu\text{M}$  IIA<sup>mtl</sup>, and 0.25% decyl-polyethylene glycol were included in the reaction mixture. 21  $\mu\text{g}$  of MtlR was first phosphorylated, as described above, for 40 min at 30 °C in 200  $\mu\text{l}$  via IICB<sup>mtl</sup> in the presence of 12  $\mu\text{M}$  [ $^{32}\text{P}$ ]PEP. Phosphorylated MtlR was bound to 50  $\mu\text{l}$  of Ni-NTA agarose and washed with 3 volumes of 0.5 ml of 25 mM Tris-HCl, pH 7.5, 0.5 mM MgCl<sub>2</sub>, 5 mM DTT, 0.25% decyl-polyethylene glycol, and 100 mM NaCl.  $^{32}\text{P}$ -MtlR was eluted in 150  $\mu\text{l}$  of wash buffer supplemented with 150 mM imidazole. 4  $\mu\text{g}$  of  $^{32}\text{P}$ -MtlR was dephosphorylated by the addition of 9 nM IICB<sup>mtl</sup> in the presence (squares) and absence (circles) of 5 mM mannitol. Samples were drawn from the reaction mixture, mixed with loading buffer, and frozen until analysis by SDS-PAGE.

equilibrium was the same for all protected areas, indicating that all of the areas are involved in the binding of the same regulator complex. The MtlR concentration at which 50% protection was observed varied from 300 to 100 nM depending on the protein sample used.

**The Effect of Phosphorylation on the Binding of MtlR to the Mannitol Promoter**—Because phosphorylation of MtlR by components of the PTS could be involved in the regulation of the activity of the regulator, we studied the influence of phosphorylation of MtlR on its affinity for its binding site. To investigate this, we performed DNA footprint experiments under various phosphorylation conditions. The difference in kinetics of the phosphorylation of MtlR by HPr and IICB, as demonstrated in Fig. 7, were used to obtain MtlR phosphorylated mainly by HPr, IICB<sup>mtl</sup>, or HPr and IICB<sup>mtl</sup>. MtlR-dependent DNA footprints were made with MtlR phosphorylated by different compounds of the PTS and are presented in Fig. 9A. The protection level of footprint FP1 was measured and is plotted against the MtlR concentration in Fig. 9B. The other protected areas, FP2 to FP5, showed MtlR concentration dependences similar to that of FP1 and are not included in Fig. 9B. Differences in the concentration dependence of the protection were observed un-



**FIG. 8. DNA footprint analysis of MtlR binding to the mannitol promoter region.** A DNA fragment containing the mannitol promoter region from position -127 to +212 was synthesized by PCR, using a  $^{32}\text{P}$ -labeled primer at the -127 position. In each reaction, 20 kcpm of the DNA probe was exposed to DNase I in the presence of 3, 1, 0.3, 0.1, 0.03, or 0.01  $\mu\text{g}$  of MtlR and analyzed on a sequence gel (lanes 1–6, respectively) (see under “Materials and Methods” for details). Position of the binding was determined by the A and G sequence ladder, obtained using the same labeled primer as was used for the probe. The protected regions, FP1 to FP5, are indicated by brackets, and hypersensitive sites HS1 and HS2 are indicated by arrows. The numbers at the left indicate nucleotide positions relative to the mannitol start site.

der different phosphorylation conditions. Reaction conditions that favor dephosphorylation of MtlR were created by adding all components of the PTS in the presence of mannitol but in the absence of PEP (reaction V). Under these conditions, 50% protection was observed at a MtlR concentration of 92 nM. When the protein was incubated with PEP, EI, and high concentrations of HPr, the affinity of MtlR for its binding site increased by a factor of 4, to 22 nM (reaction I). This indicates that phosphorylation of MtlR by HPr has a positive effect on the DNA binding properties of MtlR. Phosphorylation of MtlR by IICB, using PEP, EI, IIA, IICB<sup>mtl</sup>, and low concentrations of HPr, resulted in a 14-fold decrease in the affinity, to 1300 nM (reaction II), compared with the nonphosphorylated protein in reaction V. Clearly, phosphorylation of MtlR by IICB<sup>mtl</sup> has a negative effect on the DNA binding properties of MtlR. Phosphorylation of MtlR by both HPr and IICB<sup>mtl</sup>, using high concentrations HPr, results in a slight increase in binding, to 720 nM (reaction III), compared with MtlR phosphorylated only by IICB<sup>mtl</sup>. The strongest binding of MtlR to the DNA is observed under conditions where MtlR is phosphorylated by PEP, EI, and HPr and simultaneously dephosphorylated by IICB<sup>mtl</sup> and mannitol (reaction IV). Under these conditions, an affinity of 3.4 nM was measured that was 27 times higher than the affinity of the nonphosphorylated MtlR in reaction V and 382 times higher than the affinity of MtlR phosphorylated by IICB<sup>mtl</sup> in reaction II. It is important to emphasize that the MtlR used for these experiments had to be dephosphorylated first. Because of the rapidity of the Ni-NTA isolation procedure of the His-tagged MtlR and the use of buffers with a pH of 8, the protein, as isolated, is still phosphorylated. Consequently, the effects

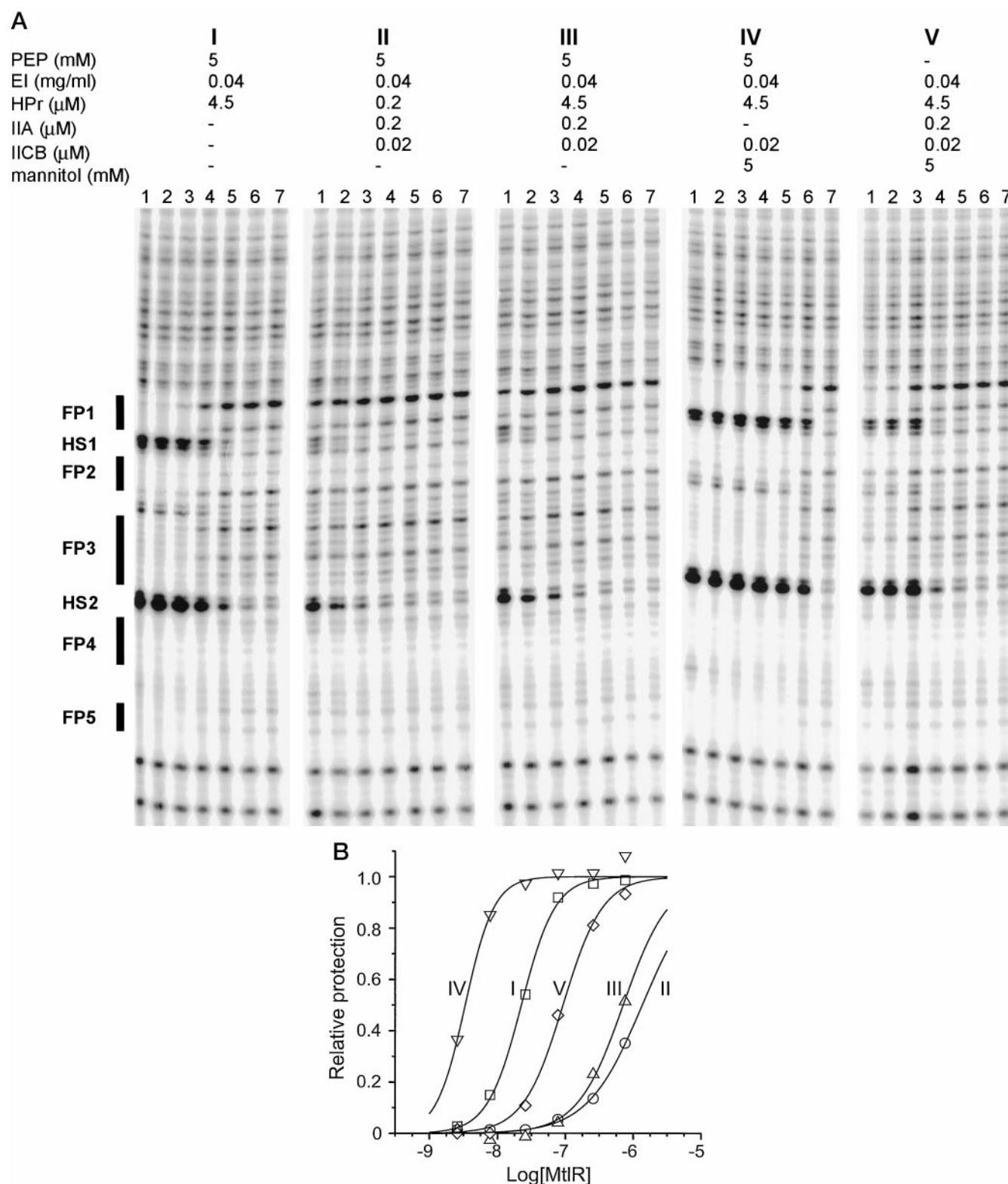


FIG. 9. The effect of phosphorylation on the binding of MtlR to the *mtl* promoter region. For each phosphorylation condition, MtlR, dephosphorylated by incubation at pH 6.5 and 30 °C for 2 h, was diluted to 1.3  $\mu$ M in phosphorylation buffer containing different combinations of PTS components. The final concentrations of these components in the mixture are listed at the top for each experiment. After 2 h of phosphorylation at 30 °C, each mixture was diluted with the same phosphorylation mixture without MtlR. An equal amount of DNA binding buffer was added to each sample, resulting in final MtlR concentrations as follows: lane 1 (of each set), 766 nM; lane 2, 255 nM; lane 3, 77 nM; lane 4, 26 nM; lane 5, 7.7 nM; lane 6, 2.6 nM; lane 7, 0.0 nM for each experiment. A, DNA footprint created of each sample, as described under "Materials and Methods." B, the increase in protection compared with lane 7 of sets I–V, containing no MtlR, was calculated from the intensities of the protected region FP1 and plotted against the logarithm of the MtlR concentration.

described above were only observed if the protein was incubated at pH 6.5, to accelerate hydrolysis of the phosphohistidines present in the isolated MtlR. The change in the level of

MtlR phosphorylation by this treatment was estimated by adding IICB<sup>mtl</sup> and [<sup>3</sup>H]mannitol and monitoring the formation of [<sup>3</sup>H]mannitol 1-phosphate.



TABLE I

*In vivo* expression of the mannitol transporter and the mannitol-1-phosphate dehydrogenase in *B. stearothermophilus*

Cells of *B. stearothermophilus* grown on mannitol, glucose, or mannitol plus glucose were collected and resuspended in 25 mM Tris, pH 8.4, and 2 mM DTT and ruptured using a French press. Cell debris was removed by centrifugation at  $3000 \times g$ , and the cytoplasmic and membrane fractions were separated by centrifugation at  $200,000 \times g$ . IICB<sup>mtl</sup> and MPDH activities were determined on the membrane and cytosolic fractions, respectively, as described under "Materials and Methods." IICB<sup>mtl</sup> and MPDH activity is expressed as nmol of [<sup>14</sup>C]mannitol-P and  $\mu$ mol of NADH formed per min per g of total protein, respectively.

Substrate	IICB <sup>mtl</sup>	MPDH
0.5%	nmol min <sup>-1</sup> g <sup>-1</sup>	$\mu$ mol min <sup>-1</sup> g <sup>-1</sup>
Glu	7.1	22
Mtl	167	300
Mtl + Glu	36	162

**The Activity of the Mannitol Promoter *In Vivo***—The intensity of the signal obtained by the primer extension experiment in Fig. 4 is dependent on the growth substrate used. Glucose lowers the intensity of the signal, indicating catabolite repression of the mannitol operon by glucose. The presence of a mannitol regulator and a CRE site already suggest that expression of this operon is induced by mannitol and repressed by glucose. To confirm this suggestion, *B. stearothermophilus* was grown on mannitol, glucose, and mannitol plus glucose. Expression levels of the first and the last gene of the mannitol operon were determined by measuring the IICB<sup>mtl</sup> and the MPDH activity, respectively (Table I). The IICB<sup>mtl</sup> activity was measured using the purified *B. stearothermophilus* EI, HPr, and IIA<sup>mtl</sup>. When grown in the presence of glucose as the only energy and carbon source, the activity of IICB<sup>mtl</sup> and MPDH are low. The activity of both enzymes increases if the cells are grown on mannitol instead of glucose by factors of 24 and 14, respectively. Expression levels found with growth on mannitol can be repressed by the addition of glucose to the growth medium. However, the extent of repression for both enzymes is not equal. The IICB<sup>mtl</sup> specific activity decreases by a factor of 4.6, whereas the MPDH specific activity decreases by a factor of 1.9 upon the addition of glucose to the medium. Involvement of a second promoter, located between *mtlA* and *mtlD*, could be an explanation for this observation. A possible position for such a second promoter could be the 284-bp gap between the *mtlA* and *mtlR* genes. Two putative promoters are assigned in this region by the method of Reese *et al.* (41).

#### DISCUSSION

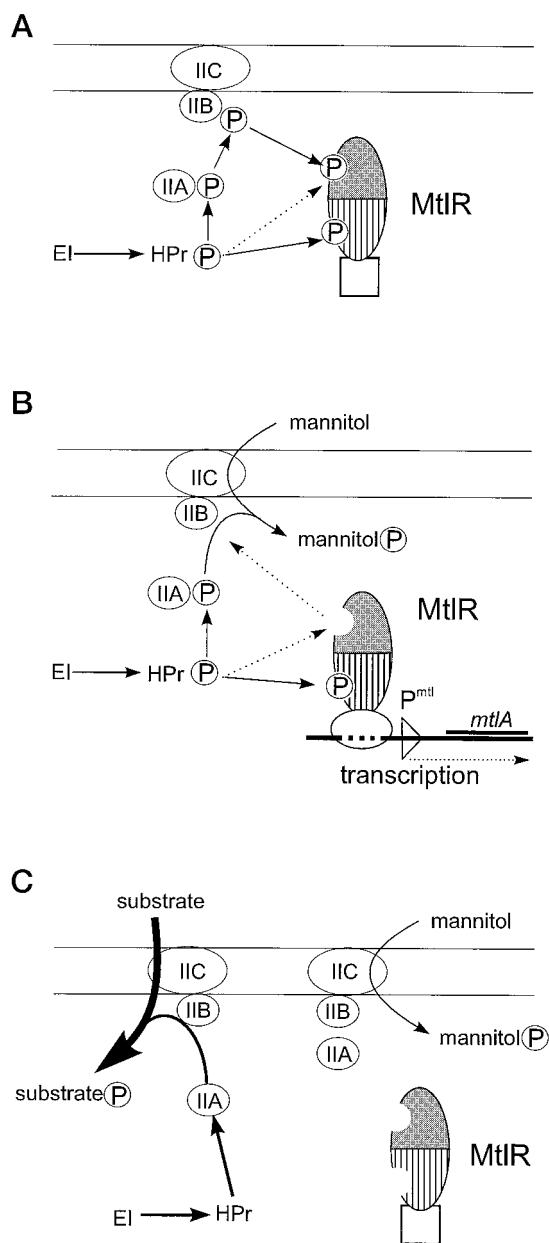
Analysis of the promoter region of the *B. stearothermophilus* operon revealed a  $\sigma^A$ -dependent promoter overlapping a CRE box. The transcription start site of this promoter was confirmed by primer extension. Binding of the CcpA repressor to the CRE box could be responsible for the observed catabolite repression of IICB<sup>mtl</sup> and MPDH by glucose, as observed for similar CRE boxes in *B. subtilis* (38). The similarity between the promoter region of the mannitol operon of *B. subtilis*, *S. carnosus*, and *B. stearothermophilus* and the presence of a MtlR-like protein in all three organisms suggests a similar mode of regulation. Binding of MtlR to a sequence upstream from the -35 box was expected based on the similarity of this region between these organisms. Indeed, DNA footprint experiments revealed that MtlR binds to this region. Five regions were protected against DNase I digestion by MtlR. It is likely that MtlR bound at this position can affect the expression of the mannitol operon by interacting with the RNA-polymerase binding to the promoter. The MtlR binding site, determined by DNA footprinting, spans a large region of 46 base pairs. This could be an indication that

two or more MtlR molecules bind to this site. Because the affinity for each of the protected areas is the same, the binding of a single complex would be more likely than the independent or cooperative binding of several monomers to this site.

MtlR can be phosphorylated both by HPr and IICB<sup>mtl</sup>, as was demonstrated *in vitro*, using purified *B. stearothermophilus* EI, HPr, and IIA<sup>mtl</sup> and membrane vesicles containing IICB<sup>mtl</sup>. The most probable targets for phosphorylation are the phosphorylation domains PRD-I and PRD-II, which are conserved in antiterminators and in LicR and LevR. The N-terminal PRD-I domain in LevR is phosphorylated by HPr, whereas the C-terminal PRD-II domain is phosphorylated by LevE, a IIB-like protein (42). Phosphorylation by HPr was also demonstrated *in vitro* for the antiterminators SacY and SacT (34, 43). Phosphorylation of SacY (44) and BglG (31) by the sugar-specific PTS proteins, SacX and BglF, respectively, has been observed. The phosphocysteine in the B-domain of BglF is the phosphoryl donor both for the carbohydrate and for BglG (45). A similar competition between mannitol and MtlR takes place in the mannitol system, because the addition of mannitol results in the dephosphorylation of MtlR by IICB<sup>mtl</sup>.

Studies on several antiterminators, as well as LevR and LicR, revealed that phosphorylation of these proteins enhance their regulating activity (recently reviewed by Stülke (30)). Transcriptional activation by LevR, SacT, and LicT is dependent on or is stimulated by the presence of EI and HPr. Phosphorylation of these proteins by HPr is most likely the activating signal. Negative control by sugar-specific components of the PTS was observed *in vivo* in the case of LevR, LicT, SacY, SacT, and BglG (32, 33, 46–49), probably caused by the phosphorylation of the regulator by one of the sugar-specific PTS components. The effect of phosphorylation on the activity of antiterminators and LevR has been determined *in vivo*, using mutants with defects in the PTS phosphorylating proteins or mutant regulators in which the putative phosphorylation sites have been replaced. Only in the case of BglG, and now also MtlR, has a direct effect of phosphorylation been observed. The phosphorylation of BglG by the  $\beta$ -glucoside transporter BglF causes dissociation of the active BglG dimer in to inactive monomers (31).

Phosphorylation of MtlR changes the affinity of MtlR for its binding site. A maximum 382-fold difference in affinity was observed under different phosphorylation conditions. Phosphorylation of MtlR by HPr results in an increase of the affinity of the MtlR for the DNA, whereas phosphorylation by IICB<sup>mtl</sup> results in a decrease. The negative effect of the phosphorylation of MtlR by IICB<sup>mtl</sup> dominates the positive effect of the phosphorylation by HPr, because the difference in affinity between MtlR phosphorylated by IICB<sup>mtl</sup> alone or IICB<sup>mtl</sup> plus HPr is minor. If MtlR is a transcriptional activator, which is regulated in a way comparable to LevR, phosphorylation of one of the PRD domains by HPr should result in an increased binding to the DNA, whereas phosphorylation of the other PRD domain by IICB<sup>mtl</sup> should result in a decreased affinity. The highest affinity of MtlR for the DNA was observed when the protein was phosphorylated by PEP, EI, and HPr and simultaneously dephosphorylated by IICB<sup>mtl</sup> and mannitol. This observation could be explained by the observation that HPr can phosphorylate at least three histidines of SacY, one on PRD-I and two on PRD-II (34). If HPr could phosphorylate a second site in MtlR, the same site phosphorylated by IICB<sup>mtl</sup>, it could reduce the activation obtained by phosphorylation of the activation site. In the presence of IICB<sup>mtl</sup> and mannitol, this second site will be dephosphorylated, allowing maximal stimula-



**FIG. 10. Proposed model for the regulation of MtlR by the PTS.** MtlR can probably be phosphorylated on at least two different domains. Phosphorylation of one domain (striped) by HPr results in an increased affinity of MtlR for the mannitol promoter region, whereas phosphorylation of another domain (gray) by IICB<sup>mtl</sup> prevents binding to this region. A, in the absence of mannitol and other substrates, all PTS proteins will be in the phosphorylated state. MtlR will be phosphorylated by HPr and IICB<sup>mtl</sup> and have a low affinity for the mannitol operator, and consequently, the expression of the mannitol operon is not stimulated. B, in the presence of mannitol, IICB<sup>mtl</sup> is dephosphorylated by transfer of the phosphoryl group to mannitol. The MtlR domain involved in the negative regulation is dephosphorylated and maximal P-HPr stimulation of MtlR binding to the operator can take place. C, when rapidly metabolizable PTS substrates, including mannitol, are transported, the concentration P-HPr decreases. MtlR is dephosphorylated at the stimulation domain, and the expression of the mannitol operon is no longer stimulated.

tion by HPr. Mutagenesis studies<sup>2</sup> indicate that both His<sup>235</sup> and His<sup>294</sup> in PRD-I and His<sup>348</sup> and His<sup>405</sup> in PRD-II are necessary for IICB<sup>mtl</sup>- and HPr-dependent phosphorylation, respectively. Based on the same studies, indications for a third phosphorylation domain have been found, that probably can be

phosphorylated by IICB<sup>mtl</sup> resulting in a decreased affinity of MtlR for the DNA. Phosphorylation of this site by HPr could be an explanation for the reduced activation by HPr in the absence of IICB<sup>mtl</sup> and mannitol.

The observations described above fit well in a regulation model already proposed for LevR by Stülke *et al.* (30, 46) and presented in Fig. 10. Regulation of the mannitol operon would occur by two different mechanisms, (i) catabolite repression, a MtlR-independent mechanism, and (ii) transcriptional activation, a MtlR-dependent mechanism. Catabolite repression is mediated by the presence of a CRE box. High levels of fructose 1,6-bisphosphate, a signal of high internal energy levels, would stimulate a kinase to phosphorylate HPr on a serine. The binding of the P-ser-HPr:CcpA complex to the CRE box would result in the inactivation of the mannitol promoter. Transcriptional regulation is suggested by the presence of MtlR. Binding of the regulator to a site upstream of the mannitol promoter could result in the activation of the expression of the mannitol operon. The affinity of MtlR for this site is regulated by phosphorylation of MtlR by the general PTS component HPr and the mannitol-specific component IICB<sup>mtl</sup>. IICB<sup>mtl</sup> acts not only as the mannitol transporter but also as a mannitol sensor. When mannitol is absent, the concentration phosphorylated IICB<sup>mtl</sup> will be high, resulting in the phosphorylation of MtlR at the IICB<sup>mtl</sup>-specific site. This causes the inactivation of MtlR as a transcriptional activator by preventing binding to the mannitol operon. However, if mannitol is present, IICB<sup>mtl</sup> will transfer the phosphate group mainly to mannitol and not to MtlR, preventing inactivation of MtlR. Phosphorylation by HPr can be considered as a CcpA-independent mechanism of catabolite repression. High uptake rates of PTS substrates, such as glucose and fructose, but also mannitol, result in decreased concentrations of P-his-HPr. MtlR will not be phosphorylated by HPr. However, if the PTS-dependent uptake activity is low, most of the HPr will be present as P-his-HPr, phosphorylate MtlR on its activation domain, and stimulate expression of the mannitol operon by binding upstream of the mannitol promoter. Whether stimulation by P-his-HPr takes place depends on the presence of mannitol, because the negative effect of phosphorylation by IICB<sup>mtl</sup> dominates the positive effect of phosphorylation by HPr on the binding of MtlR to the promoter region. The suggestion that the HPr-dependent stimulation by phosphorylation of MtlR on one site can be reduced by phosphorylation of a second site by HPr, a site that can also be dephosphorylated by IICB<sup>mtl</sup>, fits the proposed model. In this case, initial phosphorylation of MtlR by HPr will result in a reduced activation of the expression of the mannitol operon. Only when mannitol is present will the expressed IICB<sup>mtl</sup> dephosphorylate this second site and allow full stimulation by HPr.

The mechanism coupling phosphorylation and DNA binding is still uncertain. Does phosphorylation of MtlR lead to conformational changes within the protein, changing its DNA binding domain, or does it affect the aggregation state? The observed dissociation of BglG, upon phosphorylation of PRD-I by BglF, into inactive monomers (31), favors the latter explanation. In this case, the MtlR would be a multimer that binds to the DNA as one complex, because the footprints FP1 to FP5 all have the same MtlR dependence. If phosphorylation by HPr and IICB<sup>mtl</sup> influence the oligomeric state, they will affect the concentration of the active complex in the system and possibly the affinity of this complex as well.

A 1400-fold difference between the IICB<sup>mtl</sup> and HPr dependences in the rate of phosphorylation of MtlR has been observed. Differences in phosphorylation kinetics can be expected, because the concentrations of HPr and IICB<sup>mtl</sup> in the cell are very

<sup>2</sup> S. A. Henstra, R. H. Duurkens, G. T. Robillard, manuscript in preparation.

different. These differences in phosphorylation kinetics are probably also a way to tune the interaction between the phosphorylation state of the PTS components and the activity of the transcriptional regulator. The concentration of P-his-HPr and P-IICB<sup>mtl</sup> and, consequently the phosphorylation level of MtlR will be dependent on the activity of these proteins in the PTS.

The differential effect of phosphorylation, by two different proteins, on the DNA binding properties of a bacterial transcriptional regulator has not, to our knowledge, been described before. Regulation of MtlR by two components of the PTS is an example of an elegant control system sensing both the presence of mannitol and the need to utilize this substrate. More detailed studies on the number and locations of the phosphorylation sites will be necessary to elucidate the suggested negative effect of phosphorylation by HPr on a second site.

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